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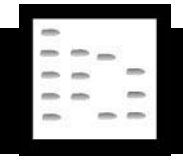
pH-ZONE REFINING COUNTERCURRENT CHROMATOGRAPHY



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What is pH-zone-refining Countercurrent Chromatography (CCC)

This countercurrent chromatography (CCC) technique distributes large quantities of ionic analytes (acids and/or bases) into a train of rectangular peaks of very high concentrations with high purity. The method utilizes ionic interaction between analytes to shift their partition coefficients according to pK_a and hydrophobicity.

Development of pH-zone-refining CCC

In liquid chromatography, isocratic elution usually produces symmetrical peaks where the peak width increases with retention time. In the course of purification of $BrAcT_3$ (*N*-bromoacetyl-3,3′,5-triiodo-L-thyronine) by high-speed countercurrent chromatography (HSCCC), it was found that the product formed an unusually sharp peak corresponding to over 2000 theoretical plates, while the preceding impurity peak showed a normal width of about 500 plates. The cause of this sharp peak formation was finally found when the collected fractions were manually analysed for pH. As shown in **Figure 1**, the pH-curve showed a gradual decline after the solvent

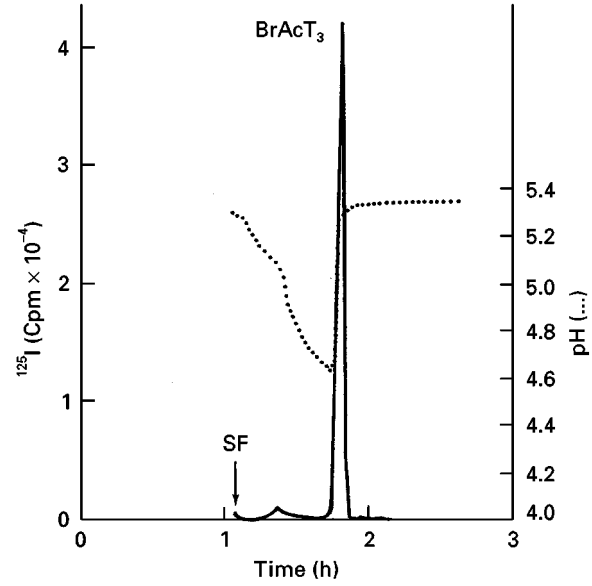

Figure 1 Disclosure of the cause of sharp peak formation by manual pH measurement. Sample: CCC-purified $BrAcT_3$ (approximately 0.1 mmol) + blank bromoacetylation mixture. The elution of the sharp peak coincides with the abrupt rise of the pH suggesting the acid in the sample solution as the cause of the peak sharpening.

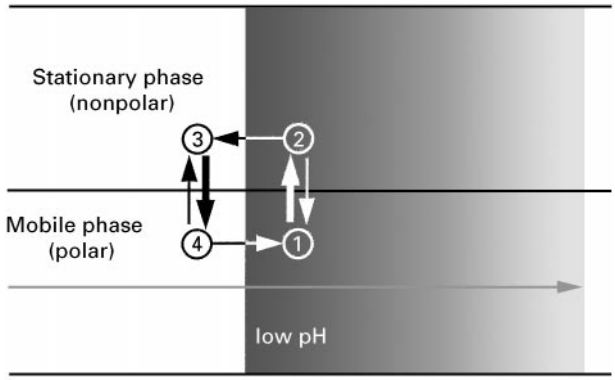

Figure 2 Schematic illustration of the peak-sharpening process in the separation column. A portion of the column contains nonpolar stationary phase in the upper half and the polar mobile phase in the lower half. The acid analyte circles around the sharp retainer border by repeating protonation and deprotonation as described in the text.

front followed by an abrupt rise which coincided with the elution of the sharp product peak. Mass spectrometric analysis of the sample solution showed the presence of bromoacetic acid, a reaction product of $BrAcT_3$ synthesis. Here, bromoacetic acid acts as a 'retainer' acid since it prevents elution of the analyte. Other organic acids such as trifluoroacetic acid (TFA) and acetic acid also can produce this effect, if introduced in the sample solution and/or the organic stationary phase. It must not be added to the mobile phase which should instead contain a base such as ammonia which acts as an 'eluter' base in the opposite sense of the 'retainer' acid.

A portion of the separation column shown in **Figure 2** indicates that the organic stationary phase is in the upper half and the aqueous mobile phase in the lower half. As described by its non-linear isotherm, the retainer acid forms a sharp rear boundary which moves through the column at a rate lower than that of the mobile phase. When the acid analyte is present in the mobile phase at position 1 (**Figure 2**), it becomes protonated due to the low pH and partitions into the organic stationary phase at position 2. As the basic mobile phase moves forward, the analyte is exposed to a higher pH at position 3 where it is deprotonated (ionized) and transferred to the lower aqueous phase at position 4. In the aqueous mobile phase the analyte migrates quickly through the sharp retainer border to repeat the cycle. Consequently, the analyte is always confined to a narrow region around the retainer border and elutes as a sharp peak together with that border.

In order to trap the analyte peak around the retainer border, one requirement must be satisfied. In **Figure 3** K_r represents the partition coefficient (solute concentration in the stationary phase divided by that in the mobile phase) of the retainer acid, and K_a and K_b for those of the analyte at acidic and basic conditions, respectively. If K_r is greater than K_a and K_b, the analyte elutes earlier than the retainer border forming a broad peak (peak 1). If K_r is smaller than K_a and K_b, the analyte elutes after the retainer border again with a broad peak (peak 3). Peak sharpening takes place only when K_r falls between K_a and K_b (peak 2).

This method allows the use of multiple retainer acids as spacers to separate sharp analyte peaks at their boundaries. **Figure 4** shows a separation of three dinitrophenyl (DNP)-amino acids by the spacer acids. The separation was performed with a two-phase solvent system composed of methyl *t*-butyl ether/water where TFA and three spacer acids were added to the upper organic stationary phase and ammonia (eluter base) to the lower aqueous mobile phase. Polar DNP-aspartic acid (DNP-asp) was eluted between acetic acid and propionic acid, DNP-alanine (DNP-ala) between propionic acid and *n*-butyric acid, and the more hydrophobic DNP-leucine (DNP-leu) after *n*-butyric acid.

This 'pH-peak-focusing' CCC has useful applications such as the concentration and detection of minor components and the improvement of analytical separations by shifting the retention time of the analyte away from non-ionic impurities. However, the most important application is found in preparative-scale separations. When the sample size of the DNP-amino acids in the above separation was in-

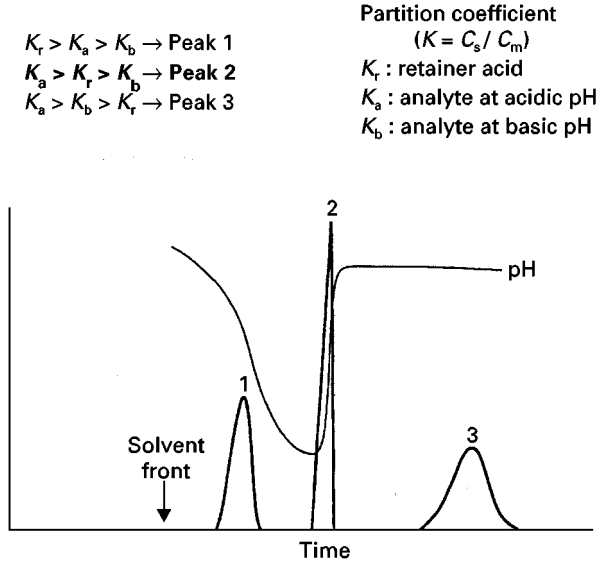

Figure 3 General requirement of sharp peak formation. Peak 1 is obtained when both K_a and K_b are smaller than K_r while peak 3 is obtained when both K_a and K_b are greater than K_r. Sharp peak 2 is formed when K_r falls between K_a and K_b, as indicated above.

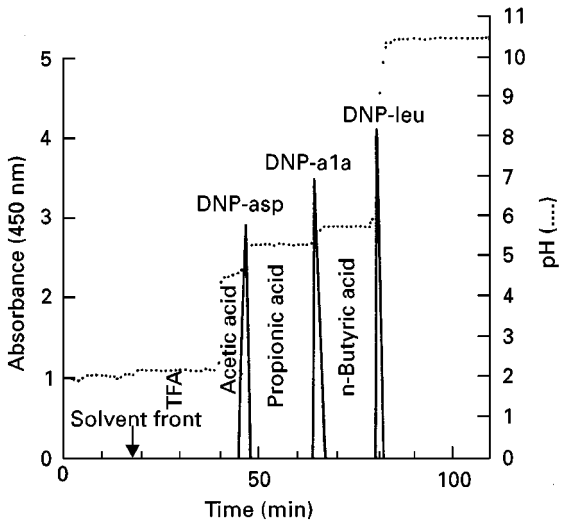

Figure 4 Separation of DNP-amino acids by spacer acids. Three spacer acids introduced in the stationary phase form pH-zones to isolate sharp analyte peaks at their boundaries. Solvent system: methyl *t*-butyl ether–acetonitrile–water (4 : 1 : 5); retainer acids: TFA, acetic acid, propionic acid, and *n*-butyric acid, each 0.4 μL mL^{-1} in the organic stationary phase; eluter base: 0.1% ammonia in the aqueous mobile phase (pH 10.77); sample: DNP-L-aspartic acid, DNP-L-alanine and DNP-L-leucine each 1 mg: flow-rate: 3 mL min^{-1}; revolution: 800 rpm; retention of the stationary phase = 81.0%.

creased by 100-fold from 1 to 100 mg, under otherwise identical conditions, the upper chromatogram shown in **Figure 5** was obtained. Each component formed a highly concentrated rectangular peak associated with a specific pH as shown by the dotted line. The elimination of the three spacer acids (Figure 5, lower) resulted in fusion of these three peaks while preserving their original rectangular shapes as demonstrated by well-defined pH-zones and K_{std} values measured with a standard solvent system (chloroform–acetic acid–0.1 M HCl at a volume ratio of 2 : 2 : 1) as shown in the lower chromatogram.

This new modification of the HSCCC method produces characteristic pH-zones according to pK_a and hydrophobicity of analytes and for this reason it has been named 'pH-zone-refining CCC'. It shares many features with displacement chromatography and provides several advantages over the conventional HSCCC technique such as increased sample loading capacity, high concentration of fractions, concentration and detection of minor components, and detection and precise localization of rectangular major peaks by monitoring the effluent pH even though the analytes have no chromophore. Of course, unlike standard HSCCC, it depends on the ability of an analytes to exist in two different forms, one hydrophobic and one hydrophilic.

Mechanism of pH-zone-refining CCC

Model Experiment

The mechanism of pH-zone-refining CCC may be demonstrated by the following model experiments. **Figure 6** shows preparation of solvent phases to initiate the experiment for separating acidic analytes. About equal volumes of ether and water are equilibrated in a separatory funnel and the two phases separated. A retainer acid such as TFA is added to the upper organic phase (shaded) while an eluter base such as ammonia is added to the lower aqueous phase. In each experiment the column is first entirely filled with the stationary phase. This is followed by injection of sample solution containing three acidic

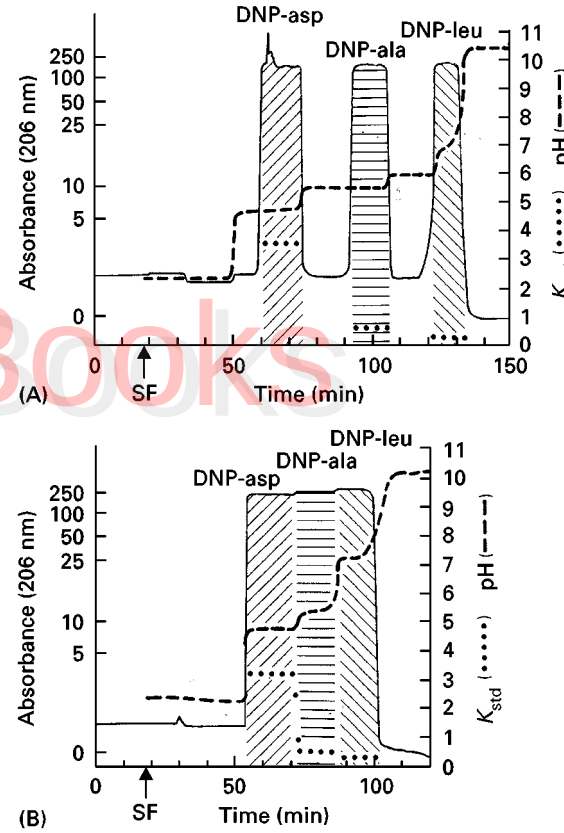

Figure 5 pH-zone-refining CCC of DNP-amino acids with (A) and without (B) spacer acid in the stationary phase. (A) Rectangular peaks of three DNP-amino acids were widely separated from each other by the spacer acids (acetic acid, propionic acid and *n*-butyric acid). (B) Elimination of the spacer acids resulted in fusion of the rectangular peaks with minimum overlapping as demonstrated by associated pH values and partition coefficient values (K_{std}). Experimental conditions are identical to those in Figure 4, except that the sample size was increased to 100 mg for each component. The K_{std} values were obtained by partitioning an aliquot of each fraction to the standard solvent system composed of chloroform–acetic acid–0.1 M HCl (2 : 2 : 1) (SF = solvent front).

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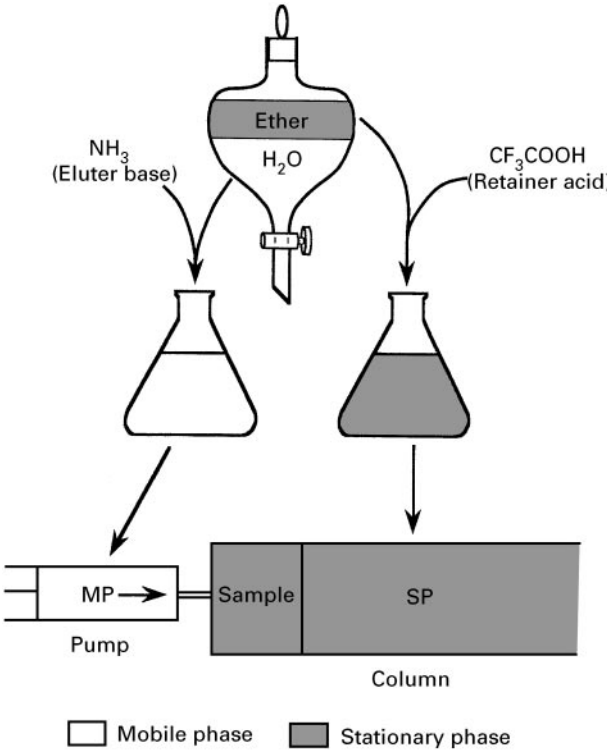

Figure 6 Model experiment for pH-zone-refining CCC for separation of carboxylic acids: preparation of solvent phases.

analytes. The mobile phase then is pumped into the column and the column rotated at a desired rate.

Figure 7 (upper diagram) illustrates the result of the experiment where a portion of the separation column shows the organic stationary phase (shaded) in the upper half and the aqueous mobile phase in the lower half. As described by its nonlinear isotherm, the retainer acid, TFA, forms a sharp boundary which moves through the column space occupied by the mobile phase at a rate considerably lower than that of the mobile phase. Three analytes, S_1, S_2, and S_3, competitively form solute zones behind the sharp TFA border according to their pK_a and hydrophobicity. S_1, with the lowest pK_a and hydrophobicity, is located immediately behind the TFA border, while S_3 with the highest pK_a and hydrophobicity is located at the end of the solute zones where it forms a sharp rear border.

As indicated by curved arrows, proton transfer takes places at each zone boundary governed by the difference in pH between the neighbouring zones. The loss of the solute from the mobile phase to the stationary phase at the zone front is compensated for by its return at the back of each zone, while ammonium ions in the aqueous phase serve as counterions for all species. After reaching equilibrium all three solute zones move at the same rate as that of the TFA border, while constantly maintaining their individual widths and pH. Charged minor components present in each zone are efficiently eliminated either forward or backward according to their partition coefficients (pK_a and hydrophobicity) and eventually accumulate at the zone boundaries. Consequently, the three analytes elute as a train of rectangular peaks with sharp impurity peaks at their narrow boundaries as illustrated in the lower diagram of **Figure 7**.

A similar experiment can be performed by using the acidified organic phase as the mobile phase. In this displacement elution mode the sharp retainer border is formed behind the solute zones, and the order of the elution for three solutes are reversed, forming a downward staircase pattern of the pH curve.

Distribution Equilibrium of Solute and Retainer Acid Within the Separation Column

Figure 8 shows the simplified distribution equilibrium of TFA (CF_3COOH) and solute S (RCOOH) between the stationary organic phase and the flowing aqueous phase on the assumption that the concentration of ionized components in the organic phase is negligible. The pH of the mobile phase in the solute zone (zone S) on the left-hand side of the sharp TFA border is given from the following three equations:

$$K_{D\text{-}s} = [RCOOH_{org}]/[RCOOH_{aq}] \quad [1]$$

$$K_s = [RCOOH_{org}]/([RCOOH_{aq}] + [RCOO^-_{aq}]) \quad [2]$$

$$K_{a\text{-}s} = [RCOO^-_{aq}]\,[H^+_{aq}]/[RCOOH_{aq}] \quad [3]$$

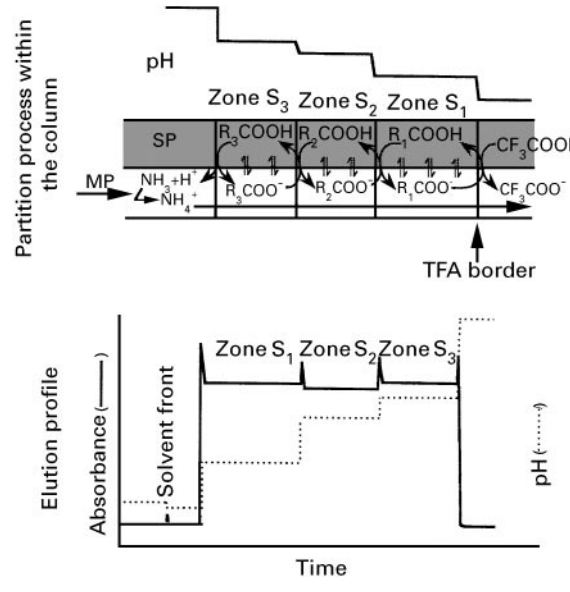

Figure 7 Model experiment for pH-zone-refining CCC for separation of carboxylic acids: partition process within the column and elution profile.

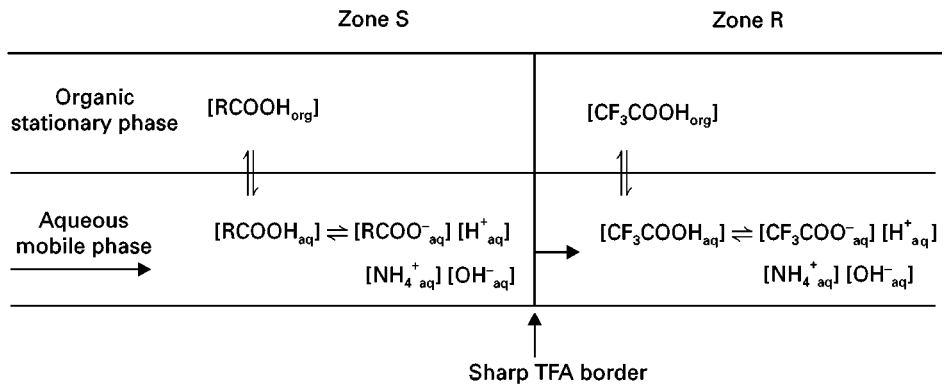

Figure 8 Distribution equilibrium of various species on both sides of the sharp TFA retainer border in the separation column.

where $K_{D\text{-}s}$ is the partition ratio of solute S (RCOOH) and $K_{D\text{-}s}$, the dissociation constant of the solute. These equations reduce to

$$pH_{z\text{-}s} = pK_a + \log\{(K_{D\text{-}s}/K_s) - 1\} \qquad [4]$$

where $pH_{z\text{-}s}$ is pH of the mobile phase in the solute zone. As shown in the above equation, the pH of the solute zone is determined by the pK_a and hydrophobicity ($K_{D\text{-}s}$) of the solute as well as its partition coefficient (K_s).

Figure 9 shows the relationship between the pH of the mobile phase and the solute partition coefficient (K) within the column (left) and the profile of pH-zones eluted (right). These curves may be drawn from eqn [4] by inserting the actual values of $K_{D\text{-}s}$ and pK_a for the solutes. If these parameters are not available, each curve can be obtained experimentally by dissolving various amounts of solute in the solvent system (containing the eluter base but no retainer acid), and measuring the pH of the lower aqueous phase and the solute concentration in both phases. Then the diagram can be constructed by plotting the pH on the ordinate against K (the solute concentration in the upper organic phase divided by that of the lower aqueous phase) on the abscissa. As described by the non-linear isotherm, K increases with increasing amounts of solute which causes a decrease in pH. In Figure 9 (left) five pH curves are arranged from the top to bottom in the order of decreasing pK_a of the solutes where the lowest curve represents that of the retainer acid with the lowest pK_a. The vertical line drawn through the critical K value, called the operating line, intersects each pH curve and determines the pH level in the corresponding solute zone eluted as shown in the diagram on the right.

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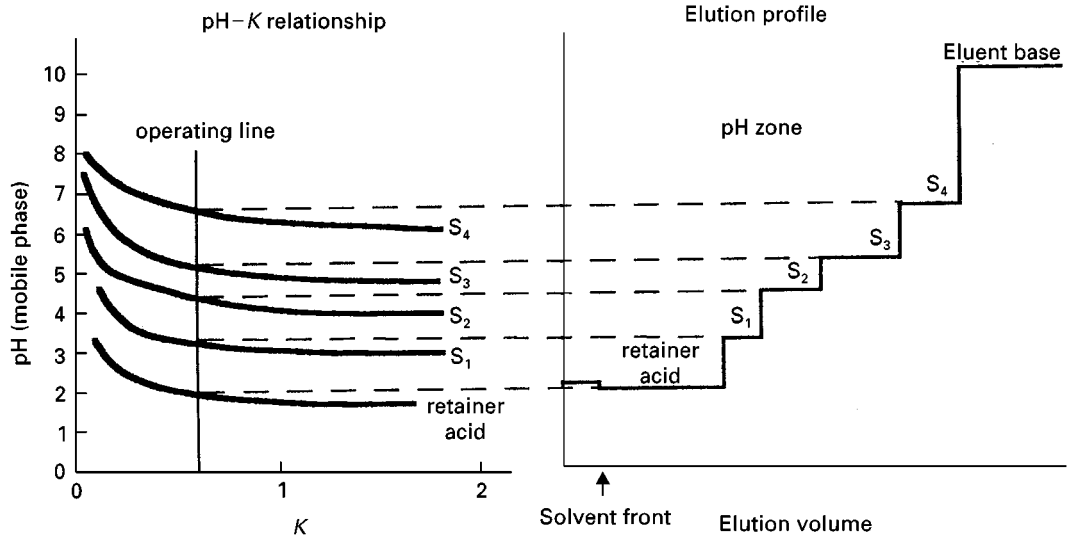

Figure 9 Relationship between pH/K curves and eluted pH-zones.

This pH vs K diagram is useful for predicting the experimental results including the order of solute elution, the pH level of each solute zone, and the feasibility of the separation. A good separation is expected from a set of pH curves which show well-separated curves with even distributions.

pH-zone-refining CCC vs Displacement Chromatography on a Solid Support

As described earlier pH-zone-refining CCC closely resembles displacement chromatography in many aspects, including formation of highly concentrated rectangular peaks, concentration of minor components at the boundaries of the major peaks and isotachic movement (moving at the same rate) of all solute zones. However, an important difference between the two is that in pH-zone-refining CCC a retainer or an eluter agent transfers the analytes from the stationary phase to the mobile phase by either protonation or deprotonation, which changes the partition coefficient of the analytes. In displacement chromatography on a solid support, the displacer displaces analytes by transferring it from the solid support to the mobile phase. Another important difference between the two methods is the concentration of the analyte in the mobile phase. In displacement chromatography, concentration of the analyte is determined by its adsorption onto the solid support. Consequently, the earlier eluting analyte shows a lower concentraction than the later eluting analytes. In pH-zone-refining CCC, on the other hand, the analyte concentration is mainly determined by the molar concentration of the counterion in the aqueous phase, and therefore all monovalent analytes are eluted at similar molar concentrations in the aqueous mobile phase. Similarities and differences between the two techniques are summarized in **Table 1**.

Application of pH-zone-refining CCC

The applications of pH-zone-refining CCC are presented here under two subheadings: standard and affinity separations. pH-zone-refining CCC separation is usually performed with a retainer agent in the stationary phase and an eluter agent in the mobile phase. However, the separation of certain groups of compounds requires an additional agent, a ligand, in the stationary phase. Such groups include enantiomers, highly polar analytes such as catecholamines and sulfonated dyes, and zwitterions such as amino acids and peptides. Samples, solvent systems and essential ingredients such as retainers, eluters and ligands used in various applications for the standard and affinity pH-zone-refining CCC techniques are summarized in **Tables 2** and **3**, respectively.

The following examples of pH-zone-refining-CCC separations have been performed using a HSCCC centrifuge equipped with a semi-analytical multilayer coil of 1.6 mm internal diameter having a total capacity of about 320 mL. In the conventional HSCCC technique the sample loading capacity of this column is limited to a few hundred milligrams.

Standard Separations

Amino acid derivatives Three chromatograms of DNP-amino acids shown in **Figure 10** demonstrate the capability of pH-zone-refining CCC. All separations were performed with the same solvent system composed of methyl *t*-butyl ether–acetonitrile–water at a volume ratio of 4 : 1 : 5 where a 200 μL quantity of TFA (retainer acid) was added to the sample solution, and 0.1% v/v aqueous ammonia (28%) (eluter base) to the aqueous mobile phase to raise the pH to 10.5.

The top chromatogram was obtained from 6 mg of the sample mixture consisting of six different

Table 1 Comparison between pH-zone-refining CCC and displacement chromatography

	pH-zone-refining CCC		*Displacement chromatography using solid support*
	Standard mode	*Displacement mode*	
Key reagents	Retainer	Eluter	Displacer
Solute transfer (phase)	Mobile → Stationary	Stationary → Mobile	Stationary → Mobile
Action location	Front of solute bands	Back of solute bands	Back of solute bands
Formation of solute bands	A train of individual solute bands with minimum overlapping		
Travelling rate of solute bands	All solute bands move together at the same rate as that of the key reagent.		
K^* in solute bands	Same as and determined by that of the key reagent for all solutes		
Impurities	Concentrated at the boundaries of the solute bands		
Peak profile	A train of rectangular peaks associated with sharp impurity peaks at their boundaries		
Solute concentration in mobile phase is determined by	Concentration of counterions in aqueous phase		Solute affinity to stationary phase
Elution order is determined by	Solute pK_a and hydrophobicity		Solute affinity to stationary phase

*K is the partition coefficient expressed by solute concentration in the stationary phase divided by that in the mobile phase.

Table 2 Samples and solvent systems applied to standard pH-zone-refining CCC

*Sample**	*Solvent systems (volume ratio)*†	*A pair of acid/base reagents*‡	
		Retainer	*Eluter*
DNP-amino acids (up to 1 g)	MBE/AcN/H_2O (4 : 1 : 5)	TFA (200 μL/SS)	NH_3 (0.1%/MP)
	MBE/AcN/H_2O (4 : 1 : 5)	TFA (0.04%/SP)	NH_3 (0.1%/MP)
	MBE/AcN/H_2O (4 : 1 : 5)	TFA, spacer acids (each 0.04%/SP)	NH_3 (0.1%/MP)
	MBE/H_2O (DPCCC)	NH_3 (22 mM/SP)	TFA (10.8 mM/MP)
	MBE/H_2O (DPCCC)	NH_3 (44 mM/SP)	TFA (10.8 mM/MP)
	MBE/H_2O (DPCCC)	NH_3 (22 mM/SP)	TFA (10.8 mM/MP) (Spacer acids/MP or SS)
Proline (OBzl) (1 g)	MBE/H_2O	TEA (10 mM/SP)	HCl (10 mM/MP)
Amino acid (OBzl) (0.7 g)	MBE/H_2O	TEA (10 mM/SP)	HCl (10 mM/MP)
Amino acid (OBzl) (10 g)	MBE/H_2O	TEA (5 mM/SP)	HCl (20 mM/MP)
CBZ-dipeptides (0.8 g)	MBE/AcN/H_2O (2 : 2 : 3)	TFA (16 mM/SP)	NH_3 (5.5 mM/MP)
CBZ-dipeptides (3 g)	MBE/AcN/H_2O (2 : 2 : 3)	TFA (16 mM/SP)	NH_3 (5.5 mM/MP)
CBZ-tripeptides (0.8 g)	BuOH/MBE/AcN/H_2O (2 : 2 : 1 : 5)	TFA (16 mM/SP)	NH_3 (2.7 mM/MP)
Dipetide-βNA (0.3 g)	MBE/AcN/H_2O (2 : 2 : 3)	TEA (5 mM/SP)	HCl (5 mM/MP)
Indole auxins (1.6 g)	MBE/H_2O	TFA (0.04%/SP)	NH_3 (0.1%/MP)
TCF (0.01–1 g)	DEE/AcN/10 mM $AcONH_4$ (4 : 1 : 5)	TFA (200 μL/SS)	MP
Red #3 (0.5 g)	DEE/AcN/10 mM $AcONH_4$ (4 : 1 : 5)	TFA (200 μL/SS)	MP
Orange #5 (0.01–5 g)	DEE/AcN/10 mM $AcONH_4$ (4 : 1 : 5)	TFA (200 μL/SS)	MP
Orange #10 (0.35 g)	DEE/AcN/10 mM $AcONH_4$ (4 : 1 : 5)	TFA (200 μL/SS)	MP
Red #28 (0.1–6 g)	DEE/AcN/10 mM $AcONH_4$ (4 : 1 : 5)	TFA (200 μL/SS)	MP
Eosin YS (0.3 g)	DEE/AcN/10 mM $AcONH_4$ (4 : 1 : 5)	TFA (200 μL/SS)	MP
Amaryllis alkaloids (3 g)	MBE/H_2O	TEA (5 mM/SP)	HCl (5 mM/MP)
	MBE/H_2O (DPCCC)	HCl (10 mM/SP)	TEA (10 mM/MP)
Vinca alkaloids (0.3 g)	MBE/H_2O (DPCCC)	HCl (5 mM/SP)	TEA (5 mM/MP)
Structural isomers (15 g)	MBE/AcN/H_2O (4 : 1 : 5)	TFA (0.32%/SP)	NH_3 (0.8%/MP)
Stereoisomers (0.4 g)	Hex/EtOAc/MeOH/H_2O (1 : 1 : 1 : 1)	TFA, octanoic acid (each 0.04%/SP)	NH_3 (0.025%/MP)
Fish oil (0.5 g)	Hex/EtOH/H_2O (4 : 1 : 5)	TFA (10 mM/SP)	NH_3 (0.1%/MP)

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*DNP: dinitrophenyl; CBZ: carbobenzoxy; OBzl: benzyl esters: βNA: napthyl amide; TCF: tetrachlorofluorescein; amaryllis alkaloids: crinine, powelline and crinamidine; vinca alkaloids: vincamine and vincine; structural isomers: 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid; Stereoisomers: 4-methoxymethyl-1-methyl-cyclohexane carboxylic acid; fish oil: mixture of docosahexaenoic acid and eicosapentaenoic acid.

†The upper organic phase was used as the stationary phase (SP) and the lower aqueous phase, the mobile phase (MP) except in DPCCC where the above relationship is reversed. MBE: methyl-*t*-butyl ether; AcN: acetonitrile; BuOH: *n*-butanol; Hex: hexane; EtOAc: ethyl acetate; MeOH: methanol; $AcONH_4$: ammonium acetate; DEE: diethyl ether; DPCCC: displacement mode.

‡TFA: trifluoroacetic acid; AcOH: acetic acid; SP: in stationary phase; MP: in mobile phase; SS: in sample solution; TEA: triethylamine.

DNP-amino acids. All components were eluted together as a sharp single peak without any visible evidence of separation. When the sample size was increased 100 times, i.e. from 6 to 600 mg, the ultraviolet (UV) trace at 206 nm produced a highly concentrated rectangular peak which was divided into six flat pH-zones (dotted line) as shown in the middle chromatogram. The partition coefficient of fractions measured with a standard solvent system composed of chloroform–acetic acid–0.1 M HCl revealed that each pH-zone corresponds to one species as indicated in the chromatogram. The bottom chromatogram illustrates the separation of 500 mg each of DNP-glutamic acid and DNP-valine under similar conditions. Each component formed a long plateau associated with its specific pH where the length of each plateau increased in proportion to the applied sample size. A sharp transition between the two plateaus indicates minimum overlap between the two peaks. A gradual decline of pH curves in both pH-zones was apparently caused by a steady increase of the travelling rate of the retainer acid border and the following solute zones through the column, since the retainer acid was added exclusively to the sample solution.

pH-Zone-refining CCC can be equally well applied to the separation of basic compounds using a retainer base such as triethylamine and an eluter acid such as hydrochloric acid. This was first demonstrated by the separation of amino acid benzyl esters. **Figure 11** shows a chromatogram of a set of amino acid benzyl esters using a two-phase solvent system composed of methyl *t*-butyl ether and water where triethylamine

Table 3 Samples and solvent systems applied to affinity pH-zone-refining CCC

*Sample**	*Solvent systems† (volume ratio)*	*A set of key reagents‡*		
		Retainer	*Eluter*	*Ligand*
(±)-DNB-leucine (2 g)	MBE/H_2O	TFA (40 mM/SP)	NH_3 (20 mM/MP)	DPA (40 mM/SP)
(±)-DNB-valine (2 g)	MBE/H_2O	TFA (40 mM/SP)	NH_3 (20 mM/MP)	DPA (40 mM/SP)
Catecholamines (3 g)	MBE/H_2O	NH_4OAc (200 mM/SP)	HCl (50 mM/MP)	DEHPA (20%/SP)
Dipeptides (1 g)	MBE/AcN/50 mM HCl (4 : 1 : 5) (SP)	TEA (20 mM/SP)		DEHPA (10%/SP)
	MBE/AcN/H_2O (4 : 1 : 5) (MP)		HCl (20 mM/MP)	
Dipeptides (1 g)	MBE/BuOH/AcN/50 mM HCl (2 : 2 : 1 : 5) (SP)	TEA (20 mM/SP)		DEHPA (30%/SP)
	MBE/BuOH/AcN/H_2O (2 : 2 : 1 : 5) (MP)		HCl (20 mM/MP)	
Bacitracins (5 g)	MBE/50 mM HCl (1 : 1) (SP)	TEA (40 mM/SP)		DEHPA (10%/SP)
	MBE/H_2O (MP)		HCl (20 mM/MP)	
FD&C Yellow No. 6 (2 g)	MBE/AcN/H_2O(2 : 2 : 3)	H_2SO_4 (0.2%/SP)	NH_3 (0.4%/MP)	TDA (5%/SP)

*DNB: dinitrobenzoyl.
†MBE: methyl *t*-butyl ether; AcN: acetonitrile; BuOH: *n*-butanol.
‡TFA: trifluoroacetic acid; NH_4OAc: ammonium acetate; TEA: triethylamine; DPA: N-dodecanoyl-L-proline-3,5-dimethylanilide; DEHPA: di-(2-ethylhexyl) phosphoric acid; TDA: tridodecylamine; SP: organic stationary phase; MP: aqueous mobile phase.

(10 mM) was added to the organic stationary phase and hydrochloric acid (10 mM) to the aqueous mobile phase. Seven components were well resolved in 3 h.

The preparative separations of three amino acid benzyl esters are shown in **Figure 12** where three sample sizes of 0.6 g (A), 3 g (B) and 6 g (C) were separated with the same solvent system composed of methyl *t*-butyl ether–water, where 5 mM triethylamine was added to the organic stationary phase and 20 mM HCl to the aqueous mobile phase. Comparison of these three chromatograms clearly shows that the increase of the sample size results in a proportional increase of the peak width whereas the width of the mixing zones remains the same as indicated by the sharp transition of the standard *K* values between the peaks (*x*-line). This again demonstrates the great potential of the technique for preparative-scale separations.

Peptide derivatives Peptides can be fractionated easily by pH-zone-refining CCC in a manner similar to that for the amino acid derivatives described above, if either amino or carboxylic terminal is blocked. **Figure 13** shows separations of a set of Z or CBZ (carbobenzyloxy)-dipeptides by pH-zone-refining CCC using a two-phase solvent system composed of methyl *t*-butyl ether–acetonitrile–water (2 : 2 : 3, v/v) with 16 mM TFA in the organic stationary phase and 5.5 mM ammonia in the aqueous mobile phase. Eight components, each 100 mg, were well resolved within 4 h. A gram-quantity separation of three components, Z-gly-gly, Z-gly-ala and Z-gly-leu, was also successfully performed with a similar solvent system.

Alkaloids Many alkaloids may be effectively separated by pH-zone-refining CCC using triethylamine in the organic phase and HCl in the aqueous phase. **Figure 14** shows chromatograms of alkaloids from a crude amaryllis extract. The separation was performed with a two-phase solvent system composed of methyl *t*-butyl ether–water. The upper chromatogram was obtained by eluting with an aqueous phase and the lower chromatogram by eluting with an organic phase (displacement mode). In both elution modes three components were well resolved. The sample size was 3 g in each separation. Elution with the organic phase yields a free base in an organic solvent which is easily evaporated. For unstable alkaloids, aqueous phase elution may be preferred because the sample is collected in the salt form that is often more stable.

Figure 15 shows a chromatogram obtained from a crude extract of *Vinca minor* by the displacement mode of pH-zone-refining CCC. Two major components, vincine and vincamine, were separated and each eluted as a free base in about 2 h. Irregularity of the pH curve was caused by instability of the pH reading obtained from the organic mobile phase.

Miscellaneous separations The method can be very useful for purification of structural isomers from a crude synthetic reaction. **Figure 16** shows a separation of 2- and 6-nitro-4-chloro-3-methoxybenzoic acids by pH-zone-refining CCC. An 11.7 g amount of the crude reaction mixture was resolved into two peaks in 3 h, the 6-nitro isomer (3.1 g) and 2-nitro isomer (5.9 g) with a mixing zone (0.7 g). One of the

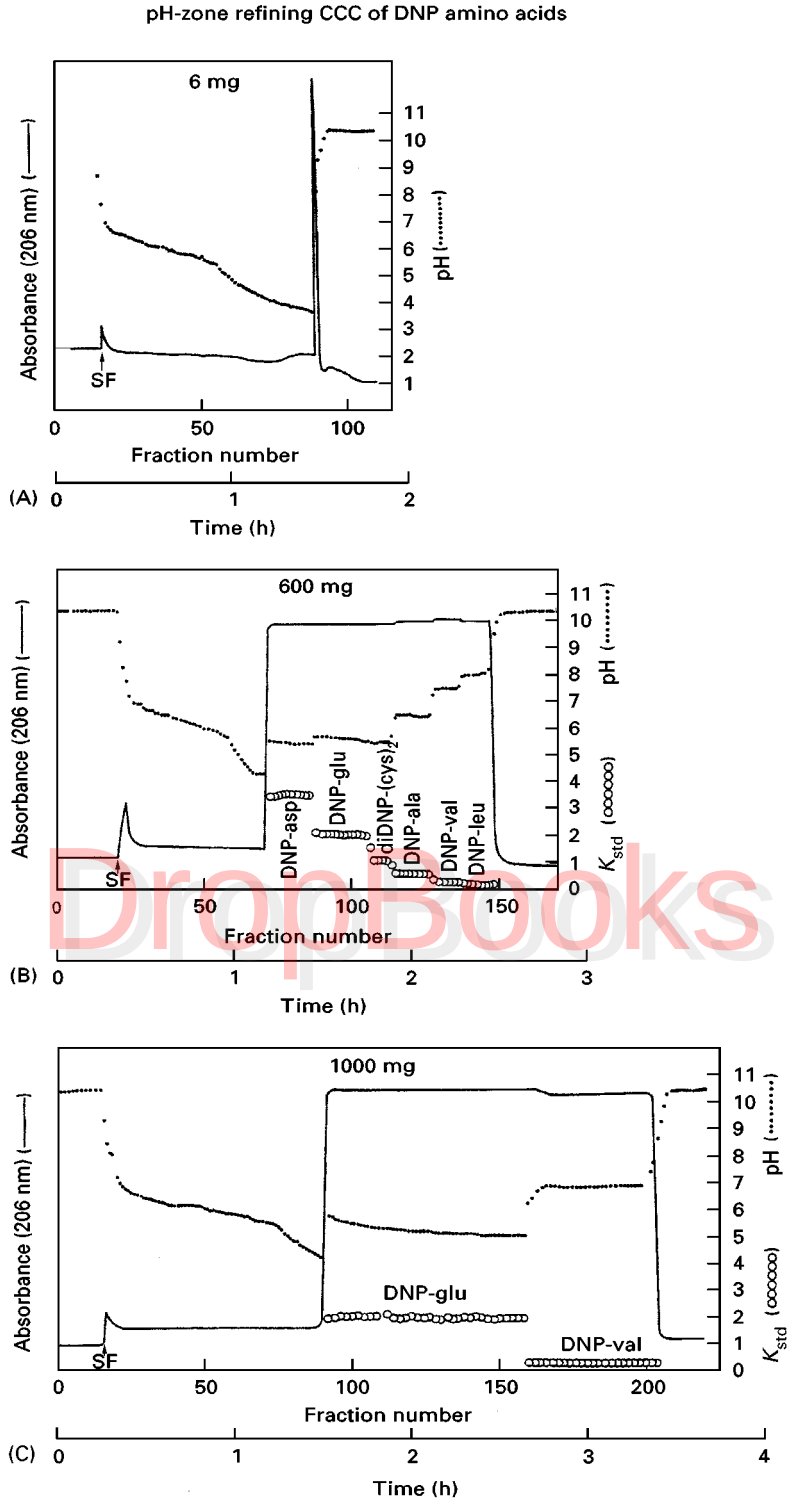

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Figure 10 Chromatogram of DNP-amino acids. Top: separation of a small amount (6 mg) of six different DNP-amino acids. Middle: separation of large amounts (600 mg) of the above six DNP-amino acids. Bottom: separation of large quantities of DNP-L-glutamic acid and DNP-L-valine (500 mg of each). SF = solvent front. Solvent system: methyl *t*-butyl ether–acetonitrile–water (4 : 1 : 5) where 0.1% aqueous NH_3 (approximately 14 mM) was added to the aqueous mobile phase (pH 10.5) and 200 μL TFA was added to the sample solution; flow-rate: 3 mL min^{-1}; detection: 206 nm; revolution: 800 rpm.

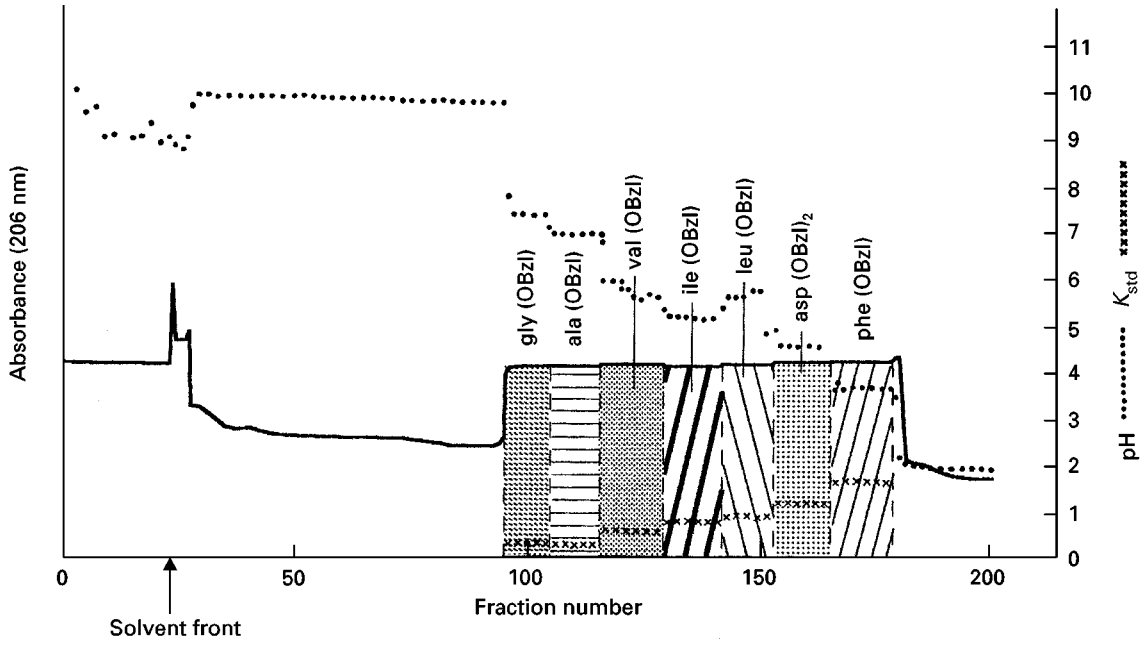

Figure 11 Separation of seven amino acid benzyl esters by pH-zone-refining CCC. Solvent system: methyl *t*-butyl ether/water, 10 mM triethylamine in upper organic stationary phase and 10 mM hydrochloric acid in lower aqueous phase; sample: a mixture of seven amino acid benzyl esters as indicated in the chromatogram, 100 mg each dissolved in 20 mL solvent; flow-rate: 3 mL min^{-1}; detection: 206 nm; revolution: 800 rpm; retention of stationary phase: 71.2%.

advantages of pH-zone-refining CCC is that compounds with no chromophore can be conveniently monitored by pH alone: the fractions of major components are located in their flat pH-zones and those of minor components at their boundaries, since in accord with eqn [4] they are unlikely to have identical or compensating pK_a and hydrophobicity. This potential is demonstrated in the seperation of the stereoisomers of 1-methyl-4-methoxymethylcyclohexane carboxylic acid, using octanoic acid as a spacer. As shown in **Figure 17**, the two isomers were eluted after the octanoic acid each forming a pH-zone with a relatively narrow mixing zone. The collected fractions were analysed by gas chromatography–mass spectrometry (GC–MS) of their esters as indicated in the upper part of the figure.

The pH-zone-refining CCC separations of hydroxyxanthene dyes and sulfonated dyes are described under separation of dyes (high-speed countercurrent chromatography).

Affinity Separations

Enantiomers In CCC, which uses no solid support in the column, the chiral selector is simply dissolved in the liquid stationary phase to carry out the separation by either conventional or pH-zone-refining CCC.

The chromatogram in **Figure 18** was obtained from 2 g of a DNB-leucine racemate using an affinity ligand, *N*-dodecanoyl-L-proline-3,5-dimethylanilide (DPA) in the stationary phase. The racemic mixture was resolved in highly concentrated rectangular peaks with minimum overlap. The fractions were analysed by analytical CCC as indicated in the diagram, using the same chiral selector, and also with CD and optical rotation instruments. This technique should be very useful in the pharmaceutical industry where an ever-increasing number of drugs are now required to be produced in chirally pure forms.

Catecholamines Catecholamines containing two or more hydroxyl groups strongly favour partition into the aqueous phase even in a polar butanol two-phase solvent system. However, the use of a ligand such as di(2-ethylhexyl)phosphoric acid (DEHPA) in the organic stationary phase radically improves their partition behaviour so that pH-zone-refining CCC of a mixture of 100 mg each of four polar catecholamines and two related compounds using the above ligand in the stationary phase can be well resolved in 3 h.

Peptides For pH-zone-refining CCC of free peptides a series of experiments was performed using DEHPA as a ligand in the stationary phase. A set of three dipeptides with a broad range in hydrophobicity was separated with a solvent system composed of methyl *t*-butyl ether, acetonitrile and water at a volume ratio

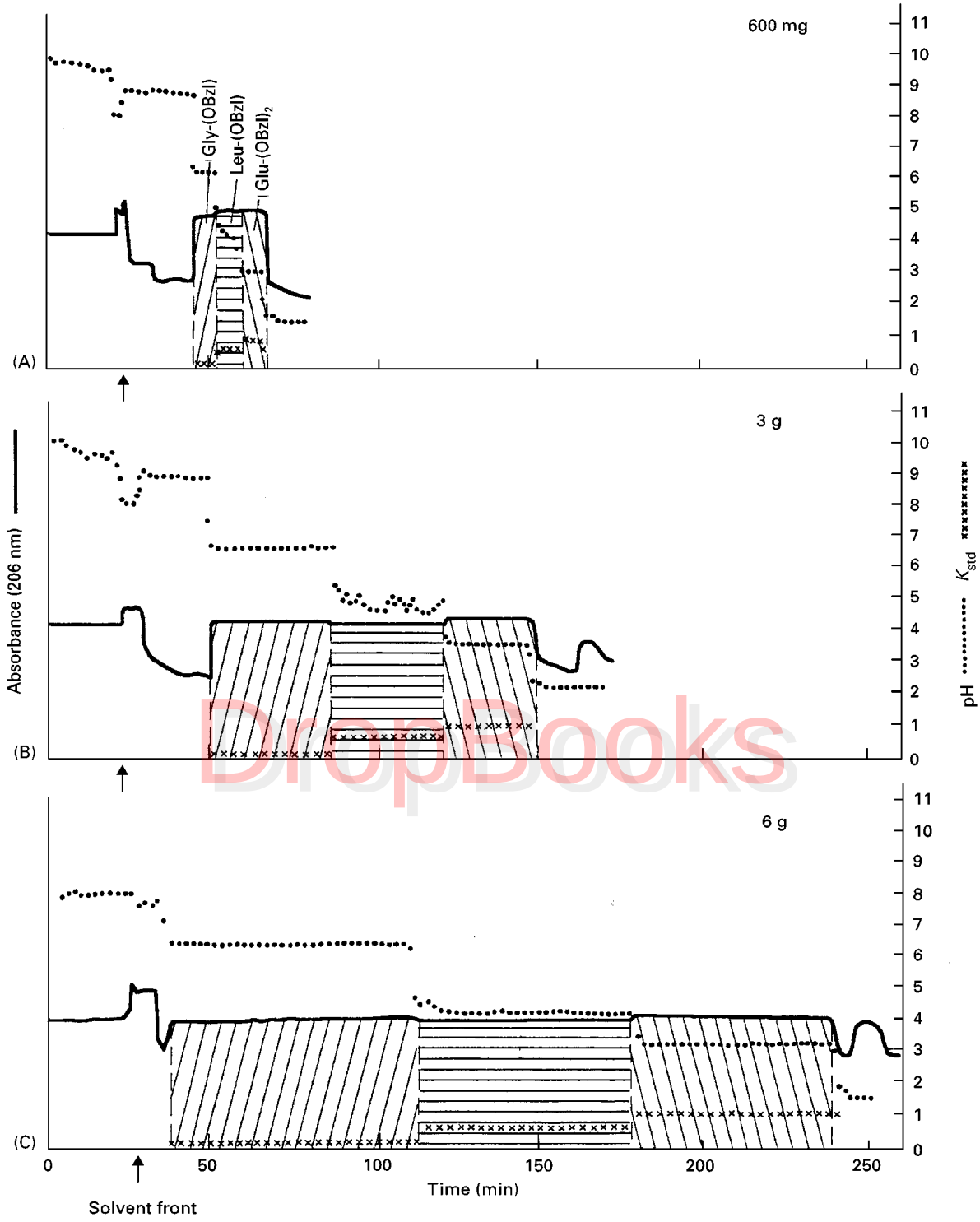

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Figure 12 Separation of three amino acid benzyl esters by pH-zone-refining CCC. Solvent system: methyl *t*-butyl ether/water, 5 mM triethylamine in organic stationary phase and 20 mM hydrochloric acid in aqueous mobile phase; sample: gly(OBzl)·Tos, leu(OBzl)·Tos and glu(OBzl)·Tos, each 0.2 g (A), 1 g (B) and 2 g (C); flow-rate: 3 mL min^{-1}; detection 206 nm; revolution: 800 rpm: retention of stationary phase: 76.5% (A), 63.3% (B) and 77.8% (C).

of 4 : 1 : 5 where triethylamine and various amount of the ligand were added to the organic stationary phase and HCl to the aqueous mobile phase. The results are shown in the left three chromatograms in **Figure 19**. At a 10% ligand concentration, the second and third peaks were fused together while the polar

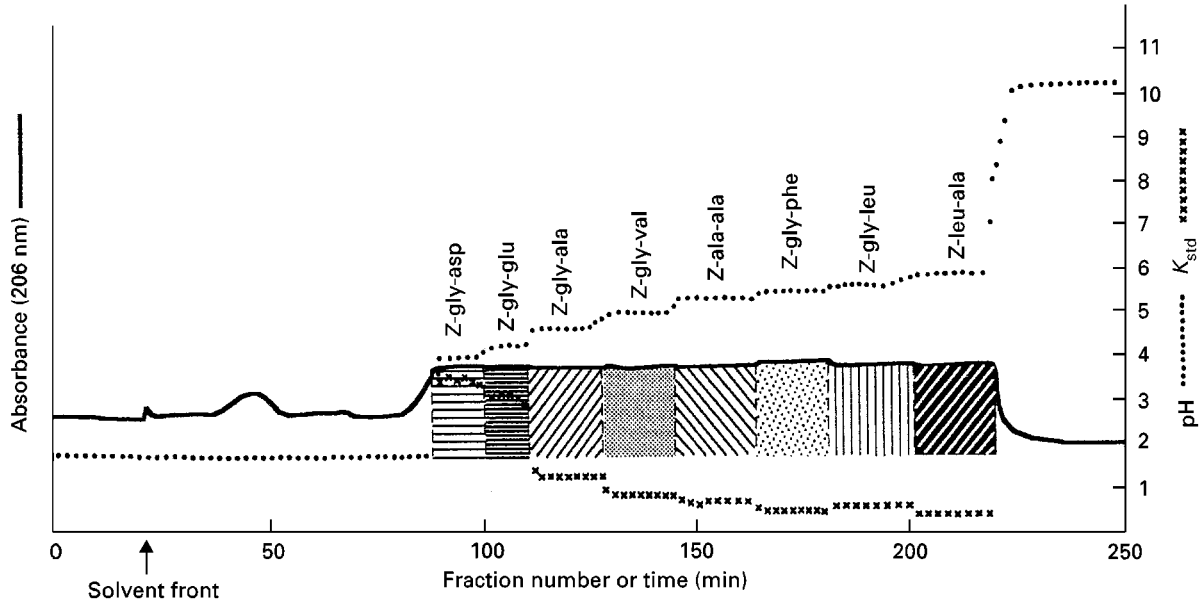

Figure 13 Separation of eight CBZ(Z)-dipeptides by pH-zone-refining CCC. Solvent system: methyl *t*-butyl ether–acetonitrile–water (2 : 2 : 3, v/v), 16 mM TFA in organic stationary phase (pH 1.83) and 5.5 mM NH_3 in aqueous mobile phase (pH 10.62); sample: eight CBZ-dipeptides as indicated in the chromatogram, each 100 mg dissolved in 50 mL solvent (25 mL each phase); flow-rate: 3.3 mL min^{-1} in the head-to-tail elution mode; detection: 206 nm; revolution: 800 rpm (first 66 mL eluted at 600 rpm to prevent the carryover of the stationary phase): stationary phase retention: 65.1%.

tyrosyl–glycine peak was eluted earlier. Increasing the ligand concentraction to 20–30% resulted in fusion of the first and second peaks while the hydrophobic tyrosyl–leucine peak was isolated and eluted much later. Increasing the polarity of the solvent system by modifying the phase composition improved the sharpness of the fused first and second peaks as shown in the right-hand chromatogram. The results

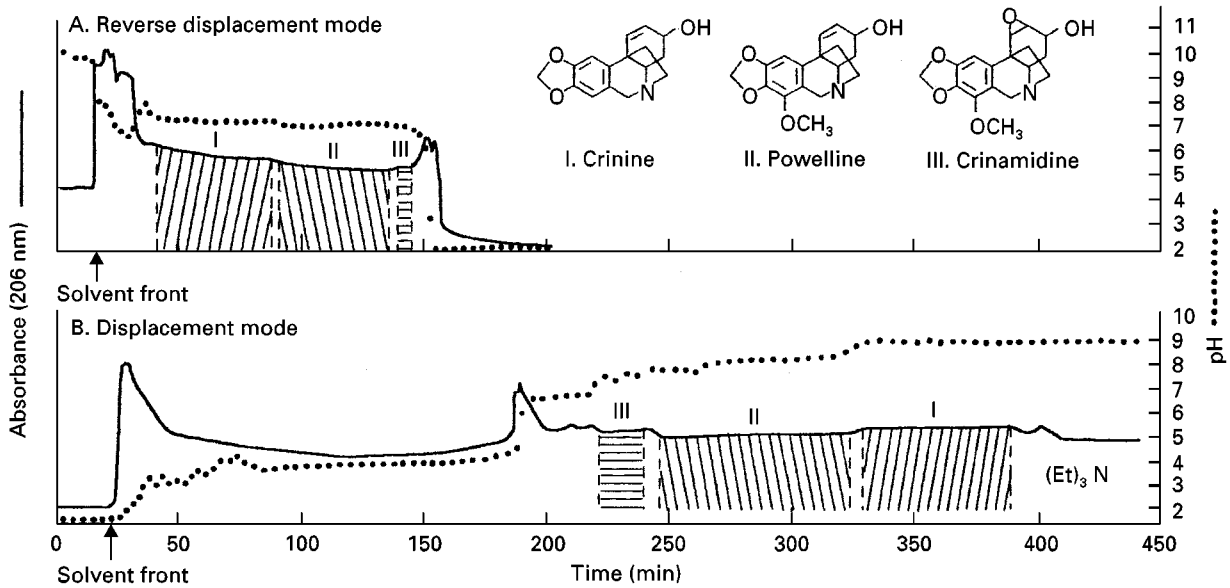

Figure 14 Chromatograms of crude alkaloid extract of solvent *Crinum moorei* obtained by standard mode (A) and displacement mode (B) of pH-zone-refining CCC. Solvent system: methyl *t*-butyl ether/water; stationary phase: (A) organic phase (5 mM triethylamine) and (B) aqueous phase (10 mM HCl); mobile phase: (A) aqueous phase (5 mM HCl) and (B) organic phase (10 mM triethylamine); flow-rate: 3.3 mL min^{-1}; sample: crude alkaloid extract of *Crinum moorei*, 3 g dissolved in 30 mL of each phase; detection: 206 nm; revolution: (A) 800 rpm (600 rpm until 66 mL of mobile phase was eluted) and (B) 600 rpm throughout.

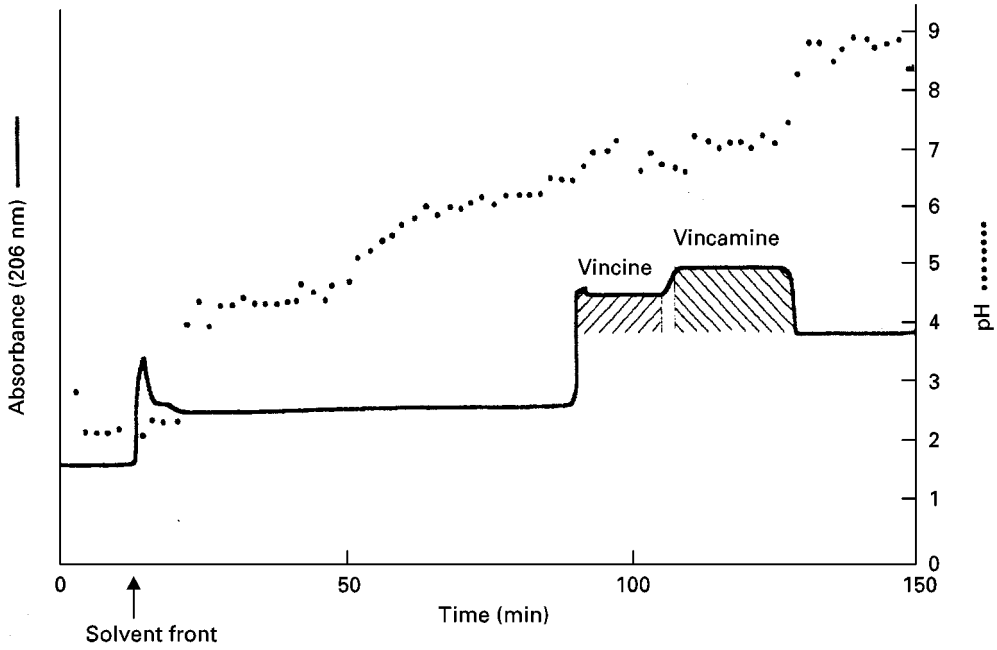

Figure 15 Separation of alkaloids from *Vinca minor* by pH-zone-refining CCC using displacement mode. Solvent system: methyl *t*-butyl ether/water, 5 mM triethylamine in organic mobile phase and 5 mM HCl in aqueous stationary phase; flow-rate: 3.3 mL min^{-1} in tail-to-head elution mode; sample: crude alkaloid extract of *Vinca minor*, 300 mg dissolved in 30 mL of solvent system (equal volumes of each phase); detection: 206 nm; revolution: 800 rpm; retention of stationary phase: 90.4%.

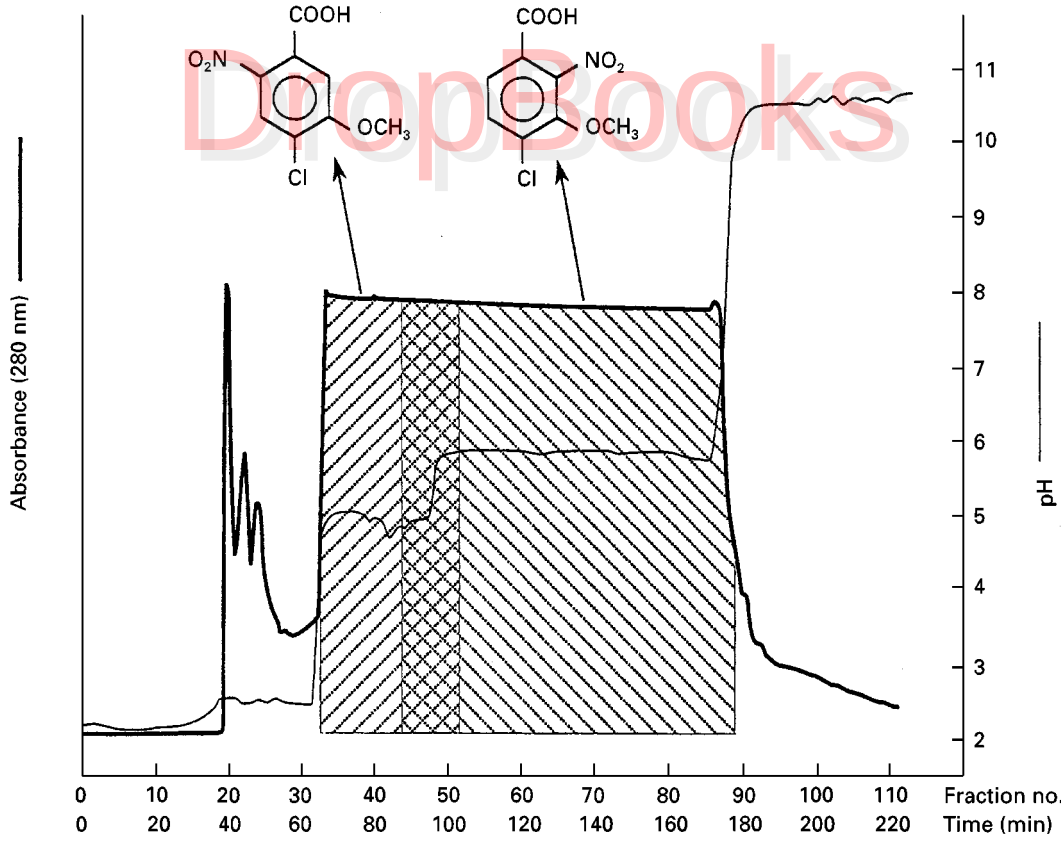

Figure 16 Separation of crude reaction mixture of 2- and 6-nitro-4-chloro-3-methoxybenzoic acids by pH-zone-refining CCC. Solvent system: methyl *t*-butyl ether–acetonitrile–water (4 : 1 : 5), TFA was added to the upper organic stationary phase at 0.3% (12 mM, pH 2.2) and ammonia 0.8% (100 mM, pH 10.6) to the lower aqueous mobile phase; sample: crude reaction mixture (11.7 g) of two isomers indicated in the figure; flow-rate: 3 mL min^{-1}; detection: 280 nm; revolution: 800 rpm.

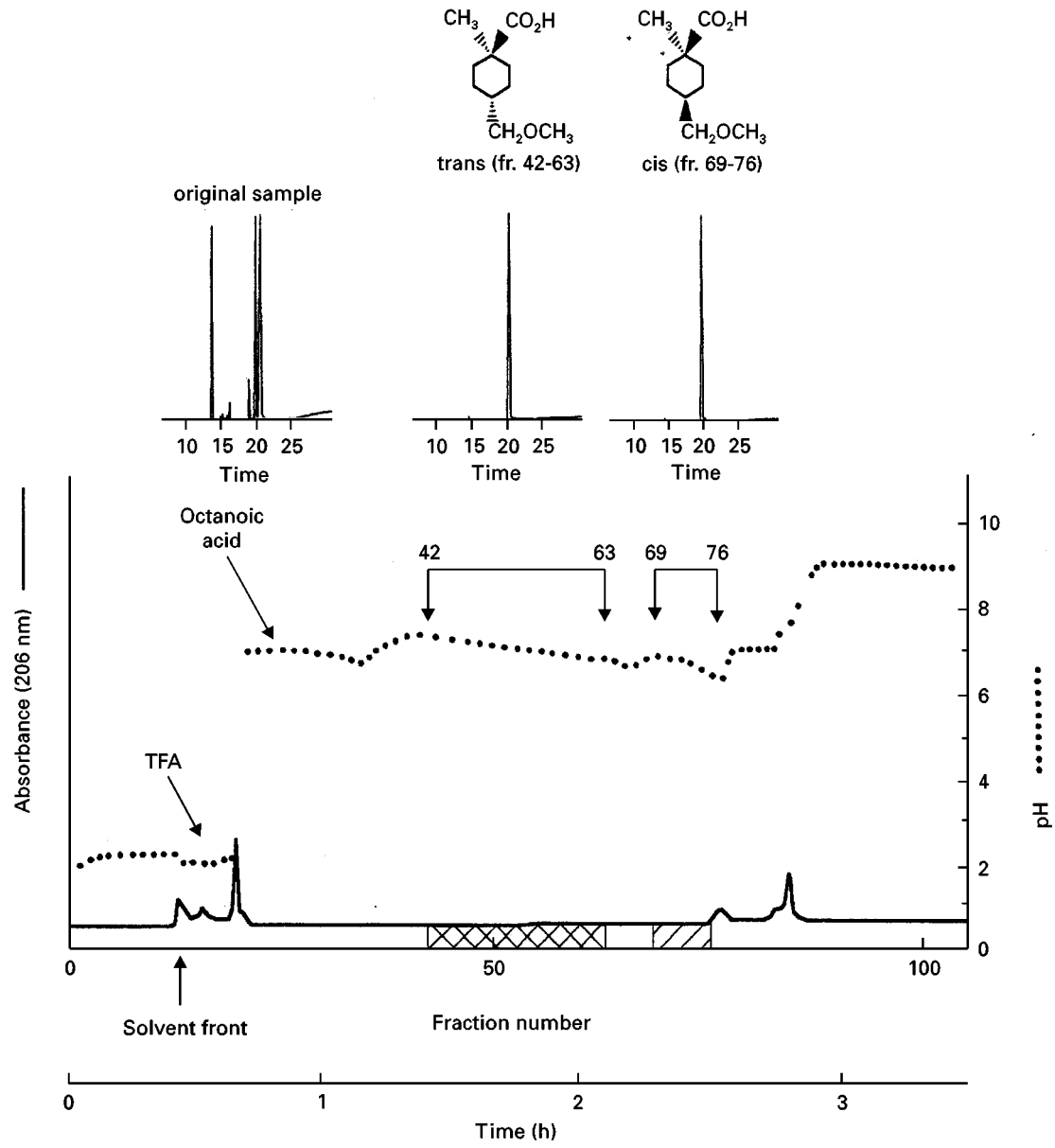

Figure 17 Separation of *trans*- and *cis*-stereoisomers of 1-methyl-4-methoxymethylcyclohexanecarboxylic acid by pH-zone-refining CCC using octanoic acid as a spacer. Solvent system: methyl *t*-butyl ether–acetonitrile–water (4 : 1 : 5), 0.32% TFA in organic stationary phase (pH 1.74), and 0.8% aqueous ammonia in aqueous mobile phase (pH 11.2); flow-rate: 3 mL min^{-1} in head-to-tail elution mode; sample: crude nitration product of 3-acetamido-4-chlorobenzoic acid (15 g) dissolved in 100 mL in equal volumes of each phase and the pH adjusted to 8.7 with aqueous ammonia; detection: 206 nm; revolution: 800 rpm (600 rpm until 66 mL of mobile phase was eluted). In analysis of fractions by GC/MS as indicated above the main chromatogram, the acids were converted to their methylesters for chromatographic purposes.

of these preliminary studies indicated that both ligand concentration and solvent composition should be adjusted according the hydrophobicity of the peptides. Under optimized conditions, pH-zone-refining CCC of dipetides was successful. As shown in **Figure 20**, both polar and non-polar groups of dipeptides, each consisting of two isomeric pairs, were well resolved in 3–4 h.

Five grams of bacitracin complex consisting of multiple components were subjected to pH-zone-refining CCC under optimized conditions similar to those applied to the hydrophobic dipeptides (**Figure 21**). The UV trace at 280 nm showed multiple peaks while the pH-curve yielded flat zones at around pH 2. As indicated by HPLC analysis, two major components, bacitracins A and F, were isolated.

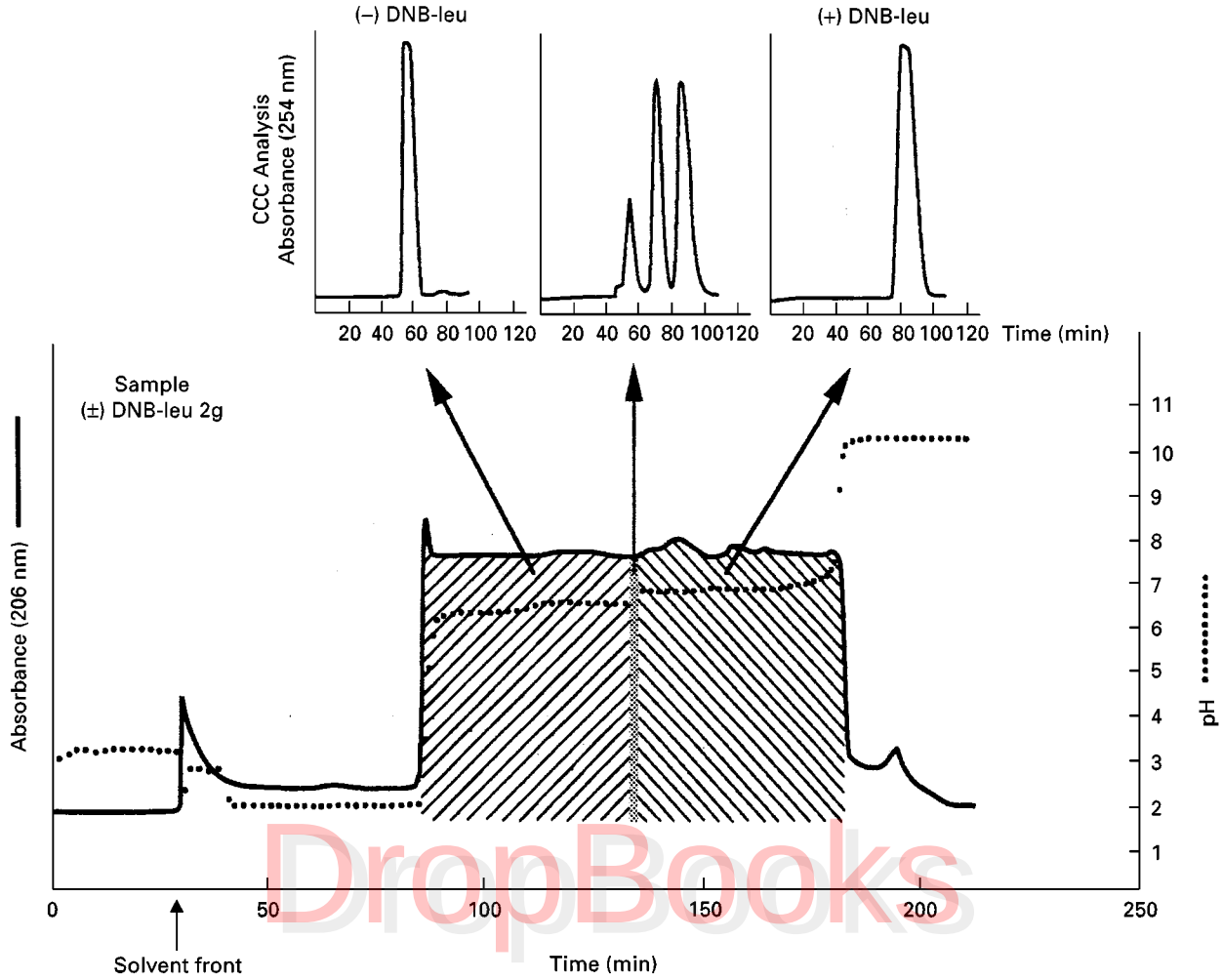

Figure 18 Chiral separation of (±)-DNB-leucine by pH-zone-refining CCC using DPA as a ligand. CCC conditions: solvent system: methyl *t*-butyl ether/water, TFA (40 mM) + ligand DPA (40 mM) in organic stationary phase and ammonia (20 mM) in aqueous mobile phase; sample: (±)-DNB-leucine 2 g; flow-rate: 3 mL min^{-1} in head-to-tail elution mode; detection: 206 nm; revolution: 800 rpm. Analytical CCC was carried out with the same column using the conventional HSCCC technique under the following conditions: solvent system: hexane–ethyl acetate–methanol–10 mM HCl (8 : 2 : 5 : 5), organic stationary phase containing DPA (20 mM); flow-rate: 3 mL min^{-1} in head-to-tail elution mode; detection: 254 nm; revolution: 800 rpm.

Detailed technical guidance for performing pH-zone-refining CCC including the choice of two-phase solvent systems, the preparation of sample solution, separation procedures, and so on has been given in the literature.

Advantages of pH-zone-refining CCC

The applications described here demonstrate the advantages of the pH-zone-refining CCC method over the conventional HSCCC technique as well as over many other commonly employed chromatographic procedures. These include:

1. Sample loading capacity is increased over 10 times for a given column.
2. Fractions are highly concentrated.
3. Increase in sample size produces a higher percentage of pure fractions.
4. Minor components are concentrated and detected at the boundaries of the major peaks.
5. Sample with no chromophore can be effectively monitored by pH.

On the other hand, pH-zone-refining CCC has the following limitations: the analytes must be ionizable and, if they have similar hydrophobicities, their pK_as should differ by 0.2 or greater. In addition, the sample size should be at least 0.1 mmol and preferably more, for each species, so it is not applicable to trace quantities. This limitation could be overcome if the column diameter were able to be sufficiently

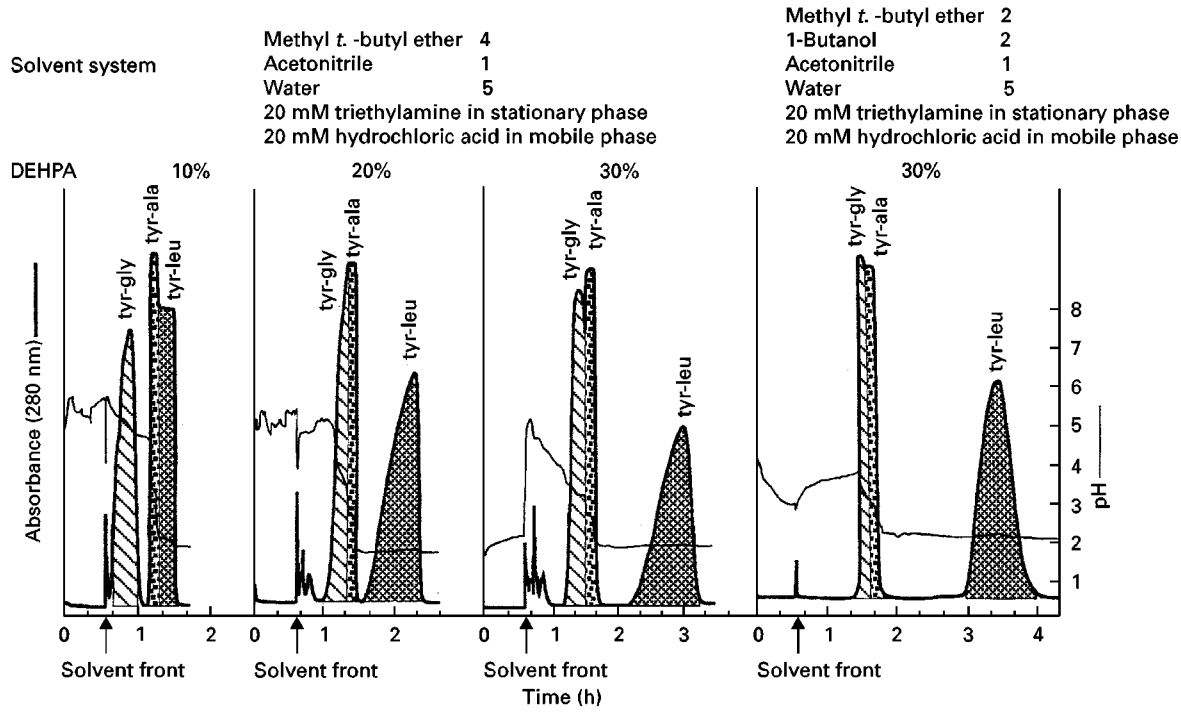

Figure 19 Separation of 3 dipeptides by pH-zone-refining CCC using DEHPA in the stationary phase. Sample: three dipeptides indicated in the chromatogram, each 100 mg; flow-rate: 3 mL min^{-1} in head-to-tail elution mode: detection: 280 nm; revolution: 800 rpm.

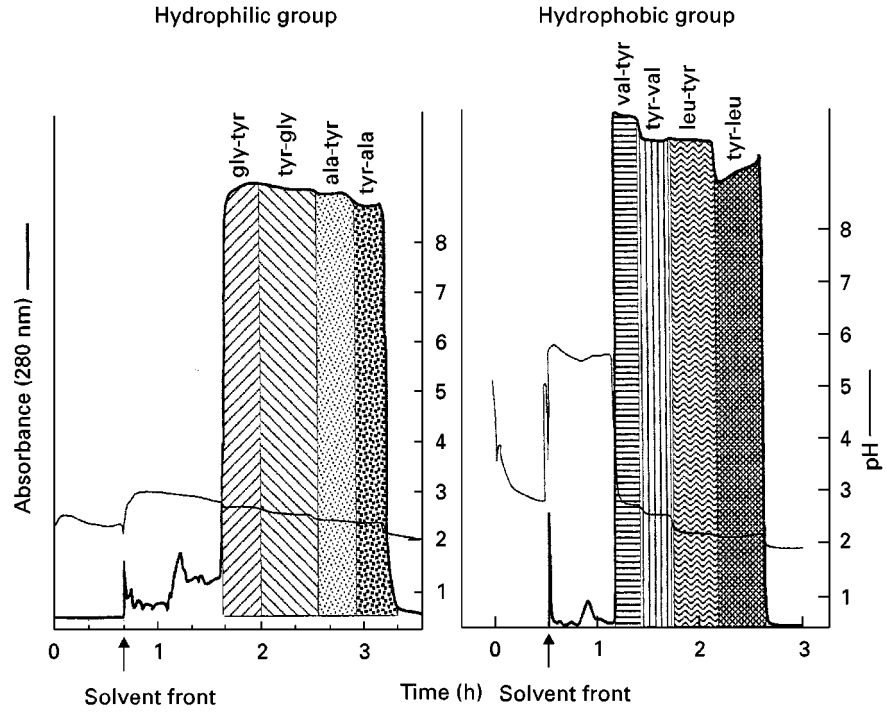

Figure 20 Separation of dipeptides by pH-zone-refining CCC. Hydrophobic (right) and hydrophilic (left) groups of dipeptides each consisting of two isomeric pairs were separated under the optimized conditions. Solvent systems: hydrophobic group: methyl *t*-butyl ether–acetonitritrile–water (4 : 1 : 5) 20 mM triethylamine and 10% DEHPA in organic stationary phase and 20 mM HCl in aqueous mobile phase, hydrophilic group: methyl *t*-butyl ether–*n*-butanol–acetonitrile–water (2 : 2 : 1 : 5) 20 mM triethylamine and 30% DEHPA in organic stationary phase and 20 mM HCl in aqueous mobile phase; flow-rate: 3 mL min^{-1} in head-to-tail elution mode; sample: dipeptides indicated in the chromatogram, total amount of 1 g for each group; detection: 280 nm; revolution: 800 rpm.

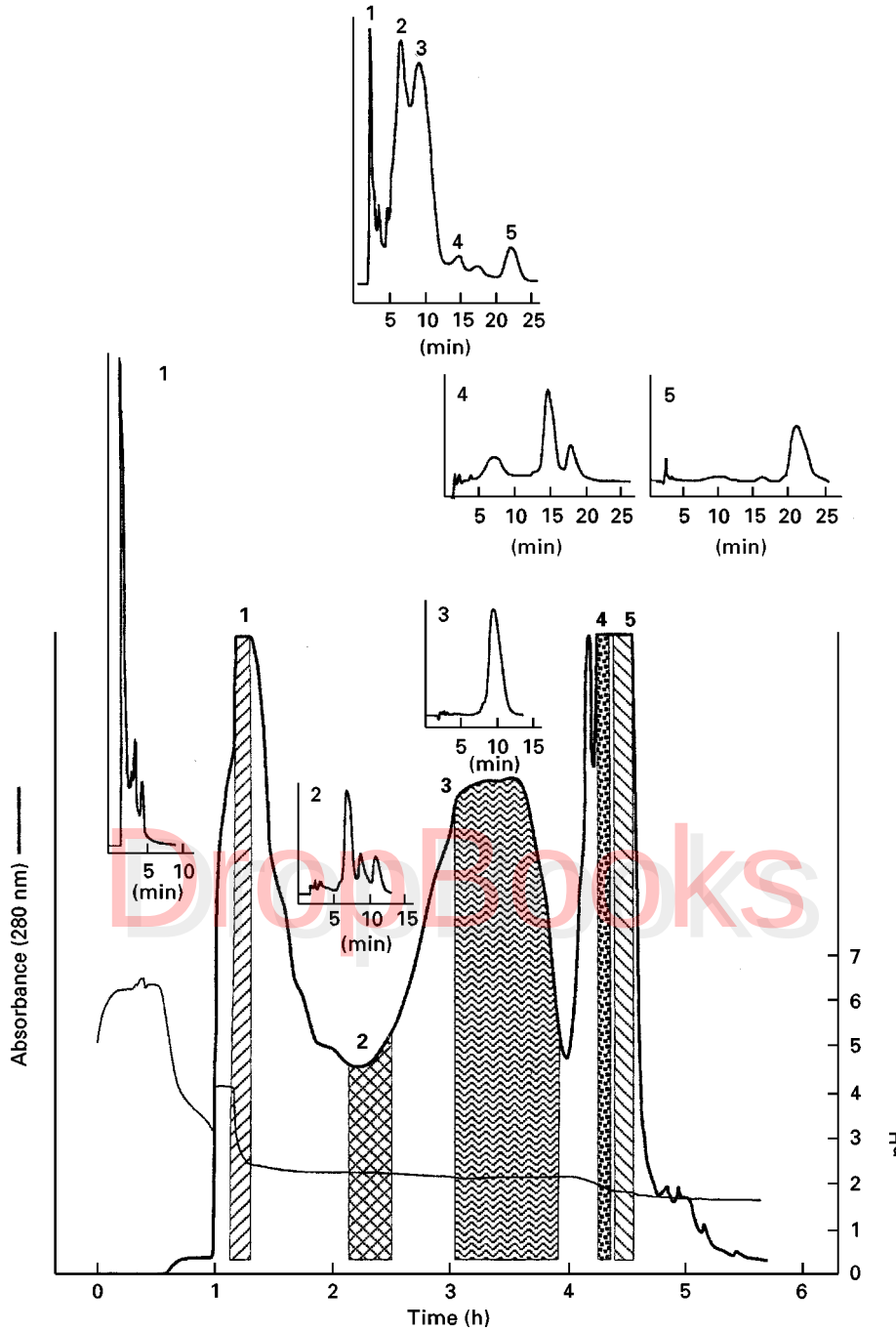

Figure 21 Preparative separation of bacitracin complex by pH-zone-refining CCC. Solvent system: methyl *t*-butyl ether–acetonitrile–water (4 : 1 : 5), 40 mM triethylamine and 10% DEHPA in organic stationary phase, and 20 mM HCl in aqueous mobile phase; flow-rate: 3 mL min^{-1}; sample: 5 g of bacitracin dissolved in 40 mL of solvent (20 mL each phase); detection: 280 nm; revolution: 800 rpm.

reduced. Of course, the technique can be used to concentrate trace quantities by the pH-peak-focusing CCC mentioned earlier.

The above features of pH-zone-refining CCC suggest that the method possesses great potential for preparative-scale research separations. Because of its unique ability to handle large-scale separations, the method should find particular application in many industrial processes.

Acknowledgement

The author is indebted to Dr Henry M Fales for editing the manuscript.

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography: Instrumentation. **Chromatography: Liquid:** Countercurrent Liquid Chromatography. **III/Alkaloids:** Gas Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Amino Acids:** Gas Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Chiral Separations. Amino Acids and Peptides: Capillary Electrophoresis. Antibiotics:** High-Speed Countercurrent Chromatography. **Chiral Separations:** Amino Acids and Derivatives; Liquid Chromatography. **Dyes:** High-Speed Countercurrent Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Ion Analysis:** High-Speed Countercurrent Chromatography. **Natural Products:** High-Speed Countercurrent Chromatography. **Proteins:** High-Speed Countercurrent Chromatography.

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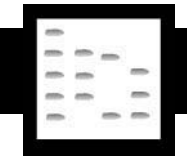
PIGMENTS



Liquid Chromatography

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Introduction

Thin-layer chromatography (TLC) represented a key development in aquatic sciences because it enabled the routine separation and quantitation of algal chlorophylls, carotenoids and their breakdown products (**Table 1** and **Figure 1**) to be obtained. These pigments can be used as markers for algal taxa, processes such as grazing or cell senescence and water masses. However, TLC methods are not easy to automate, are difficult to use in field situations and have gradually given way to liquid chromatography (LC) methods.

High performance LC (HPLC) analysis of pigments has been developed over the last 20 years. Both normal-phase (NP) and reversed-phase (RP) techniques have been used, with preference for the RP mode due to the relatively low polarity of the analysed compounds. The NP mode is now mostly used for the separation of specific pigments (e.g. monovinyl from divinyl chlorophylls) while RP-HPLC is preferred when a complete separation of all major chlorophylls and carotenoids is required. The analysis of phycobiliproteins, the other major group of algal pigments, is not yet done by chromatographic separation on a routine basis.

Within the various RP-HPLC methods, gradient elution has generally been preferred over isocratic for full pigment separation. Ion-pairing reagents, or phase buffering, have been included in a number of techniques to improve the resolution of the more polar pigments. Three groups of pigments present particular difficulties in their separation: the pigment pair lutein–zeaxanthin, the various members of the chlorophyll *c* group and the monovinyl and divinyl forms of chlorophyll *a* and *b*. Improved separation of zeaxanthin and lutein has been achieved using a nonend-capped C_{18} column (see below) and a combination of acetonitrile, methanol and ethyl acetate as mobile phase. Resolution of chlorophyll c_3 from the other compounds in the chlorophyll *c* group has been obtained by including an ammonium acetate buffer in the initial methanol mobile phase. Separation of all three forms of chlorophyll *c* has been achieved on a polyethylene column using aqeous acetone as mobile phase, as well as by using a very high ion strength solvent in combination with a high carbon loaded